REMARKS

Claims 1-3, 7, 8, 14, 15, 17, 18, 20, 22-24, 34, 37, 43-45, 55, and 56 have been amended herein. Claims 62 and 63 have been canceled herein. Claims 26-33, 38-40, 46-53, and 59-61 were previously canceled.

The amendment to Claims 2, 3, 14, 15, and 44 (inserting the designations "skin" tryptase and "lung" tryptase in place of β-I and β-II tryptase, respectively) enjoys verbatim support in the specification at page 3, lines 1-4. As noted in the specification, these designations are accepted in the relevant field as descriptions of different isoforms of tryptases. The remaining changes to the claims are supported by the claims as originally filed. No new matter is added.

Claims 1-25, 34-37, 41-45, and 54-58 and 62-63 remain pending in the application.

Favorable reconsideration is respectfully requested.

Allowable Subject Matter:

Applicants note with thanks the indication that Claims 7, 8, 17, 18, 43-45, 54-58, 62, and 63 contain allowable subject matter. Claims 7, 8, 17, 18, and 43 have been amended from dependent claims to independent claims that include all of the limitations of their respective base claims plus any intervening dependent claims. Applicants therefore submit these independent claims, and the claims dependent thereon, are now in condition for allowance.

Please note that Claims 17 and 18 as amended are identical in scope to Claims 62 and 63 as previously submitted. Thus, Claims 62 and 63 have been canceled from the application.

Supplemental Election of Species Requirement:

Applicants note with thanks the Examiner's withdrawal of the supplemental restriction requirement.

Objections to Claims 7, 17, 34-37 and 56-58:

Claims 7, 17, 34, 37 and 56 have been amended per the Examiner's recommendations. Applicants submit that this objection has now been overcome. Withdrawal of the same is respectfully requested.

Rejection of Claims 1-6, 9-12, 20-25, and 34-36 Under 35 USC §112, Second Paragraph:

This rejection is believed to have been overcome by appropriate amendment to the claims, in accordance with the Examiner's recommendations.

Specifically, Claim 1 has been amended to recite the positions of the mutated amino acid residues with respect to the polypeptide sequences shown in Fig. 1 (which corresponds to SEQ. ID. NO: 52) as well as to the various mutations presented in the Sequence List at SEQ. ID. NOS: 6, 9, 21, 23, 25, 27, 37, 39, 41, and 43.

Claims 2 and 3 have been amended to recite "skin tryptase" and "lung tryptase" in place of "β-I tryptase" and "β-II tryptase," respectively. As suggested in the passage at the top of page 3 of the specification as filed, the nomenclature of tryptases is undergoing changes as the nature of the tryptase isoforms is further elucidated. At present, the older terms "skin tryptase" and "lung tryptase" are still widely encountered in the literature. However, these terms are generally being supplanted by the terms "α" and "β," and various sub-isoforms falling under the "β" designation have been identified.

Despite the flux surrounding the nomenclature of tryptases, Applicants respectfully submit that the ordinarily skilled practitioner is fully aware of what is meant by the terms "skin tryptase" and "lung tryptase" on one hand, and "β-I typtase" and "β-II tryptase" on the other. These designations are accepted terms of art. See, for example, Peng et al. (2003) "The heterogeneity of mast cell tryptase from human lung and skin," *Eur. J. Biochem.* 270:270-283, a copy of which is attached hereto and incorporated herein as Exhibit A. As noted in the paper, there has been a vigorous scientific effort to elucidate the heterogeneity of tryptases. A general proposition supported by Exhibit A is that there is a recognizable distinction between tryptases isolated from skin tissues versus those

isolated from lung tissues. Therefore, to clarify the scope of Claims 2 and 3, Applicants have adopted in the present amendment the terminology "skin tryptase" and "lung tryptase" in place of the designations "β-I typtase" and "β-II tryptase." Claims 14, 15, and 44 have been amended in a similar fashion. See also the discussion at page 7, first full paragraph of the specification (regarding the similarity between β-I and β-II tryptases).

Claim 20 has also been amended to clarify that the claim is directed to a method of producing an **inactive** proteolytic tryptase through the mutation in the DNA encoding the tryptase. Applicants note that the mutation (as recited in the base claim, Claim 1) falls within the catalytically active site (*i.e.*, "the active site") of the corresponding enzyme. The original wording of Claim 20 was meant to convey the point that the desired mutation falls within the "active site," thus rendering the resulting enzyme "inactive." Applicants however, recognize the incongruity of using the designation "active site" when referring to a method of producing an "enzymatically inactive" protein. The amendment to both Claims 1 and 20 are believed to rectify this situation.

(For sake of completeness, however, note that the term "active site mutant" is explicitly defined in the specification at the bottom of page 11. There it is noted that an "active site" refers to a site that is required for enzymatic activity. Thus, Claim 20 is directed to mutant having a change within an active site that thus renders the resulting protein an inactive form of tryptase.)

In light of the various amendments to the claims, withdrawal of the rejections under 35 USC §112, second paragraph, are respectfully requested.

Rejection of Claims 1-6, 9-16, 19-25, 34-37, and 41-42 Under 35 USC §112, First Paragraph (Written Description):

This rejection is believed to have been overcome, in part, by appropriate amendment to the claims, and is, in part, respectfully traversed.

With respect to Claims 1-6, 9-12, 20-25 and 34-36 (all of which depend from Claim 1 or otherwise incorporate the subject matter of Claim 1), this rejection is believed to have been overcome by appropriate amendment. Specifically, Claim 1 has been amended to

provide a precise location of the relevant mutations within the ultimate protein expressed by the construct. For example, within Claim 1, the relevant mutation locations are now designated with reference both to Fig. 1 and to SEQ. ID. NOS: 6, 9, 21, 23, 25, 27, 37, 39, 41, and 43. Note that all of these sequences were included with the application as filed. See Fig. 1 as originally filed and also the specification at page 6, first and second full paragraphs.

Note that the amino residue numbering for the top amino acid sequence shown in Fig. 1 (which corresponds to SEQ. ID. NO: 52) **does not** correspond to the mature amino acid sequences shown in SEQ. ID. NOS: 6, 9, 21, 23, 25, 27, 37, 39, 41, and 43 because the sequence shown in Fig. 1 includes the amino acid residues of the secretion signal, but starts numbering the residues at the start of the mature peptide. In short, the residue numbering shown in Fig. 1 starts with the "I" (isoleucine) designated by the triangle (▼). Thus, the "H" (histidine) that is residue 44 according to Fig. 1 corresponds to residue 48 of each of SEQ. ID. NOS: 6, 9, 21, 23, 25, 27, 37, 39, 41, and 43. The "D" (aspartic acid) that is residue 91 according to Fig. 1, corresponds to residue 95 of each of SEQ. ID. NOS: 6, 9, 21, 23, 25, 27, 37, 39, 41, and 43. The "S" (serine) that is residue 194 according to Fig. 1, corresponding to residue 198 of each of SEQ. ID. NOS: 6, 9, 21, 23, 25, 27, 37, 39, 41, and 43. (To make the concordance complete, please also note that the full sequence shown in the top line of Fig. 1 is also presented as SEQ. ID. NO: 52; thus residues 44, 91, and 194 of Fig. 1, are the same as residues 74, 121, and 224 of SEQ. ID. NO: 52.) Regrettably, the situation is made slightly more convoluted because the PatentIn software renders amino acid sequences in 3-letter code, while Fig. 1 is rendered in 1-letter for sake of brevity.

In terms of the written description requirement of §112, first paragraph, the genus of expression constructs recited in Claim 1 is thus disclosed verbatim in the application as filed, as evidenced by the sequences depicted in Fig. 1, and recited in the Sequence List, most notably at SEQ. ID. NOS: 6, 9, 21, 23, 25, 27, 37, 39, 41, 43 and 52. See also the list of suitable promoter/terminators at page 18, last paragraph of the specification. See also Table 1 (starting at page 35 of the specification) which lists the various mutants that

were actually fabricated, inserted into a construct as now claimed, cloned into a yeast host, and the mutant tryptases encoded thereon expressed. Some of these tryptases were even isolated and characterized. See the Examples, especially starting at page 40. Applicants therefore submit that with respect to Claims 1-6, 9-12, and 34-36, this rejection has been overcome.

With respect to Claims 13-16, 19, 37, 41, and 42 this rejection is respectfully traversed. Specifically addressing Claims 13-16, 19, and 37, each element recited in Claim 13 (the base claim) is clearly and concisely described in the specification to the level required by the first paragraph of §112.

In support of this rejection, the Office states, at the top of page 7 of the Office Action, that in the present circumstance "There is no structural feature which is representative of all of the members of the genus of polynucleotides encoding any active/inactive proteolytic tryptase...." Applicants respectfully submit that the Office is reading limitations out of Claim 13. The construct recited in Claim 13 positively requires a DNA sequence "encoding proteolytic tryptase having **an active site mutation**." Emphasis added. The terms "active site mutant" and "active site" are explicitly defined in the specification at the bottom of page 11. As noted there, the active sites of proteolytic tryptases are amino acids 44, 91, and 194 as illustrated in the representative tryptase illustrated in Fig. 1. Thus, Claim 13 **does not** encompass "all of the member of the genus of polynucleotides encoding any active/inactive proteolytic tryptase." Claim 13 does, however, encompass the genus of proteolytic tryptases having an active site mutation as defined in the specification.

On this point, Applicants respectfully submit that the specification contains a rather extensive written description of all aspects of the invention recited in Claim 13 and the claims dependent thereon. Isolation of the native tryptase DNA is described at page 16; incorporation of that DNA into an expression construct is described at page 17, line 25 of the specification. Note that the starting plasmid, pPIC9, is commercially available from Invitrogen (see page 18, line 10). The various promoter/terminators that can be used in the invention are listed at page 18, last paragraph of the specification.

Transformation of eukaryotic hosts is described starting at page 21 of the specification. Converting β-I tryptase to β-II tryptase is described at page 23, line 20. (For what it's worth, the inconsistency of tryptase nomenclature in the prior art is noted at page 13, lines 7-8 of the specification.)

Most notably, however, are the Examples beginning at page 32 of the specification. Table 1 at page 35 of the specification describes a host of actual mutants that were made and which fall within the scope of Claim 13. These mutants were transformed in yeast hosts and the resulting tryptase was isolated and several were extensively characterized. See the discussion beginning at page 40 of the application as filed.

In short, the specification contains a great deal of written description both in the general sense about how to fabricate constructs according to Claim 13, as well as explicit working examples of how to make, express, and characterize the mutant proteins. Thus, Applicants submit that the rejection of Claims 13-16, 19, and 37 as failing to comply with the written description requirement of §112, first paragraph is untenable.

Claims 41 and 42 are drawn to constructs that drive the expression of "mature proteolytic tryptases" having enzymatic activity. This portion of the rejection is traversed because the phrase "mature proteolytic tryptase" is explicitly defined at the paragraph spanning pages 12 and 13 of the specification. Thus, these claims do not encompass all constructs that express any enzymatically active tryptase. The tryptase encoded by the construct must be enzymatically active without any "post-expression or post-isolation chemical processing." See the very last lines of page 12 of the specification. In short, the constructs recited in Claims 41 and 42 encode tryptases that self-assemble into enzymatically active tetrameric enzymes without any further intervention on the part of human hands. Applicants respectfully submit that the passages from the specification cited above clearly provide an ample written description of this class of polynucleotides.

Applicants therefore submit that as applied to Claims 13-16, 19, 37, 41, and 42, this rejection is improper. Withdrawal of this portion of the rejection is respectfully requested.

Rejection of Claims 1-6, 9-16, 19-25, 34-37, and 41-42 Under 35 USC §112, First Paragraph (Enablement):

Applicants submit that this rejection has been overcome, in large part, by the amendments to the claims as discussed in the prior sections. Specifically, Claim 1 has been amended to recite the positions of the relevant locations for the mutation with reference to both Fig. 1 and the Sequence List.

In the same fashion as in the written description rejection, the Office states, at page 9 of the Office Action, that the claims require a polynucleotide encoding "any" active or inactive trypatse. This simply is not the case. The construct recited in Claim 13 positively requires a DNA sequence "encoding proteolytic tryptase having **an active site mutation**." The terms "active site mutant" and "active site" are explicitly defined in the specification at the bottom of page 11. As noted there, the active sites of proteolytic tryptases are amino acids 44, 91, and 194 as illustrated in the representative tryptase illustrated in Fig. 1. Thus, Claim 13 **does not** encompass "all of the member of the genus of polynucleotides encoding any active/inactive proteolytic tryptase." Claim 13 does, however, encompass the genus of proteolytic tryptases having an active site mutation as defined in the specification.

The specification contains an extensive and clearly enabling description of all aspects of the invention recited in Claim 13 and the claims dependent thereon, including:

isolating the native DNA (p. 16);

incorporating the DNA into a suitable expression construct (pp. 17-18);

transformating the construct into a eukaryotic host cell (p. 21);

producing active site mutants of the native tryptase DNA (p. 30)

a host of working Examples (starting at p. 32)

Examples showing isolation of the mutant tryptase (p. 40)

Examples showing characterization of the isolated mutant tryptase (p. 42)

enzyme assays comparing the kinetics and inhibition of the mutant tryptase (pp. 44-45); and

enzyme assays revealing the ability of the mutant tryptases to cleave fibrinogen (p. 45).

Most notably, of course, are the Examples beginning at page 32 of the specification. Table 1 at page 35 of the specification describes a host of actual mutants that were made and which fall within the scope of Claim 13. These mutants were transformed in yeast hosts and the resulting tryptase was isolated and several were extensively characterized. See the discussion beginning at page 40 of the application as filed.

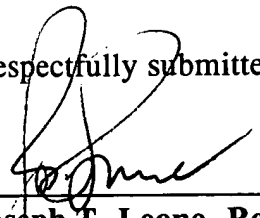
In short, the enablement requirement of §112 requires only that Applicants describe how to make and use the invention commensurate in scope with the claim language. Applicants submit that this has been done by describing a general approach for making active site mutants of various tryptases and exemplifying several working versions of the mutatns. For these above reasons, Applicants submit that this rejection under §112, first paragraph (enablement) is improper. Withdrawal of the rejection is respectfully requested.

CONCLUSION

In light of the above amendments and accompanying remarks, Applicants submit that the application is now in condition for allowance. Early notification of such action is earnestly solicited.

The Commissioner is hereby authorized to charge any additional fees or credit any overpayment to Deposit Account No. 18-2055.

Respectfully submitted,



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The heterogeneity of mast cell tryptase from human lung and skin

Differences in size, charge and substrate affinity

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Summary

There has long been conjecture over the degree to which there may be structural and functional heterogeneity in the tetrameric serine protease tryptase (EC 3.4.21.59), a major mediator of allergic inflammation. We have applied 2D gel electrophoresis to analyze the extent, nature, and variability of this heterogeneity in lysates of mast cells isolated from lung and skin, and in preparations of purified tryptase. Gels were silver stained, or the proteins transferred to nitrocellulose blots and probed with either tryptase-specific monoclonal antibodies or various lectins. Tryptase was the major protein constituent in mast cell lysates, and presented as an array of 9–12 diffuse immunoreactive spots with molecular masses ranging from 29 to 40 kDa, and pI values from 5.1 to 6.3. Although the patterns obtained for lung and skin tryptase were broadly similar, differences were observed between tissues and between individual donors. Lectin binding studies indicated the presence of mono-antennary or bi-antennary complex-type oligosaccharide with varying degrees of sialylation. Deglycosylation with protein-*N*-glycosidase F (PNGase F) reduced the size of both lung and skin tryptase, while incubation with PNGase F or neuraminidase narrowed the pI range, indicating variable degrees of glycosylation as a major contributor to the size and charge heterogeneity. Comparison of different purified preparations of lung and skin tryptase revealed no significant difference in pH profiles, but differences were seen in reactivity towards a range of chromogenic substrates, with substantial differences in K_m, k_{cat} and degree of cooperativity. Mathematical modeling indicated that the variety in kinetics parameters could not result solely from the sum of varying amounts of isoforms obeying Michaelis–Menten kinetics but with

different values of K_m and k_{cat}. The heterogeneity demonstrated for tryptase in these studies suggests that there are important differences in tryptase function in different tissues.

Keywords: mast cell; tryptase; glycosylation; lectin; 2D gel electrophoresis.

Abbreviations: Con A, concanavalin A; DFP, diisopropyl fluorophosphate; FBS, fetal bovine serum; <Glu-, l-pyroglutamyl-; MAA, *Maackia amurensis* agglutinin; MEM, minimal essential medium; MeOCO-, N^{α}-methoxycarbonyl-; MUGB, 4-methylumbelliferyl-*p*-guanidinobenzoate; PHA-L, phytohemagglutinin-L; Pip-, pipecolyl-; PNGase F, protein-*N*-glycosidase F; SNA, *Sambucus nigra* agglutinin; SNP, single nucleotide polymorphism; Suc-, N^{α}-succinyl-; WGA, wheat germ agglutinin

Introduction

Tryptase (EC 3.4.21.59) is a serine protease of mast cell origin with trypsin-like substrate specificity [1,2]. Upon activation of these cells with allergen or other stimuli, it is released along with other potent mediators of inflammation including other neutral proteases, histamine, proteoglycans, eicosanoids and cytokines. Its actions on peptides [3,4], proteins [5,6], cells [7-11] and tissues [12,13] are consistent with a pro-inflammatory role in allergic disease, and inhibitors of tryptase have proved efficacious in animal and human models of asthma [14,15].

Although tryptase is generally referred to as a single enzyme, heterogeneity has been observed at both the structural [16-20] and functional [21,22] level of the protein. Unusually for a serine protease, tryptase exists as a tetramer of approximately 130 kDa [23]. The earliest reports on this enzyme indicated microheterogeneity of the subunits, with molecular masses ranging from 31 to 38 kDa on SDS/PAGE gels, sometimes as a broad, diffuse band, sometimes as discrete bands. Both high and low molecular mass forms have been found to possess an enzymatically active site capable of being labeled by [^{3}H]diisopropyl fluorophosphate ([^{3}H]DFP) [17], while Western blotting with various antibodies has demonstrated extensive antigenic similarities [19,24]. Treatment with protein-*N*-glycosidase F (PNGase F) reduced the apparent molecular mass of the subunits in tryptase purified from pituitary [18] and from skin [20], but not from lung [16,18]. Differences in reactivity towards synthetic peptide substrates and inhibitors have been reported between tryptase purified from lung and that purified from skin [21] (although a subsequent comparison has failed to confirm such differences [25]). Functional differences were also noticed between two isoforms of lung tryptase which cleaved high molecular weight kininogen and vasoactive intestinal peptide at different sites and at different rates [22].

Initially, four different cDNA sequences were identified, α- and β-tryptase from a human lung mast cell library [26,27] and tryptases I, II and III, from a skin library [28]. Tryptase II and β-tryptase were found to be identical and to share 98% identity with tryptases I and III, but only 90% with α-tryptase. Consequently, tryptases I, II, and III have been considered together as the β-tryptases but distinguished as βI, βII, and βIII. Subsequent genomic sequencing has identified additional tryptase-like genes which have been designated γ-, δ-, and ϵ-tryptases [29-32], but these do not appear to be secreted by mast cells: γ-tryptase (also known as *trans*-membrane tryptase) is membrane-bound [30,31], δ-tryptase (also known

as mMCP-7-like protease) appears to be a pseudogene [30,33,34], and ϵ-tryptase is a product of fetal lung epithelial cells [32]. In contrast, most preparations of tissue mast cells contain ample mRNA encoding both α- and β-tryptases [35]. α-Tryptase appears to be released constitutively from mast cells as the pro-form while the β-tryptases are stored and subsequently released in the mature form on anaphylactic degranulation [36,37]. Data accruing from the Human Genome Project indicate that the four secreted mast cell tryptases, α, βI, βII, and βIII, are confined to two genetic loci with α and βI competing allelically at one locus and βII and βIII competing allelically at the other [30,34].

All four deduced amino acid sequences predict a polypeptide chain of approximately 27.5 kDa, so the experimentally observed subunit molecular masses of 30–38 kDa are indicative of extensive post-translational modification. Consistent with these observations is the presence of two consensus N-glycosylation sites in α- and βI-tryptase, and one such site in βII- and βIII-tryptase [27,28]. Interestingly, a single nucleotide polymorphism (SNP) has been reported for βII-tryptase which would result in two glycosylation sites in a significant proportion of the population [38]. The application of 2D gel electrophoresis and subsequent Western blotting to lysates of purified skin mast cells revealed multiple forms of tryptase with major differences in size and charge, together with evidence for variable glycosylation [20]. However, this sensitive analytical procedure has not been employed to characterize tryptase from the lung or other sources, or to compare tryptase from different tissues or donors.

The importance of tryptase as a major mediator of allergic disease, and its potential value as a target for therapeutic intervention call for a more detailed understanding of the forms of tryptase in human tissues. In the present studies we have applied 2D gel electrophoresis with Western blotting to examine the size and charge heterogeneity of tryptase from lysates of purified lung and skin mast cells and have employed lectin binding studies to investigate the nature of glycosylation. In addition, we have purified tryptase from both lung and skin tissues, and have compared the kinetics of cleavage of a range of chromogenic substrates.

Materials and methods

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Isolation of lung mast cells

Human lung mast cells were isolated as described previously [39]. Briefly, cells from macroscopically normal human lung tissue (obtained through surgical resection for lung cancer) were dispersed using collagenase (type 1A, 1.0 $mg \cdot mL^{-1}$), hyaluronidase (type 1, 0.75 $mg \cdot mL^{-1}$), protease (type A, 0.5 $mg \cdot mL^{-1}$), bovine serum albumin (BSA, 25 $mg \cdot mL^{-1}$) and penicillin/streptomycin solution (25 $\mu L \cdot mL^{-1}$; all from Sigma, Poole, UK) at 37 °C for 75 min with agitation, suspended in MEM/FBS (minimal essential medium/fetal bovine serum; Gibco BRL, Paisley, UK), and centrifuged on 65% isotonic Percoll (Sigma) at 750 g for 20 min at 4 °C to remove erythrocytes. Cells were harvested above the erythrocyte pellet, and further purified using affinity magnetic selection with an antibody (YB5.B8) specific for a mast cell-specific surface marker (c-*kit*) coupled to Dynabeads (Dynal). Kimura staining indicated that the purity of mast cells thus obtained ranged from 65% to 95% of all nucleated cells.

Isolation of skin mast cells

Mast cells were isolated as described previously from infant foreskin tissue obtained at circumcision of children [39,40]. Cells were dispersed enzymatically in MEM/FBS and mast cells were purified by density sedimentation through a discontinuous gradient of 60, 70 and 80% isotonic Percoll (density 1.076–1.100 $g \cdot mL^{-1}$) at 500 *g* for 20 min at 4 °C. Cells were pooled from the bottom of the gradient and the 70–80% interface. These suspensions consisted of 70–98% mast cells.

Enzyme purification

Tryptase was purified from high salt extracts of homogenized human lung tissue (obtained *post mortem*), or skin tissue (removed from amputated limbs) using cetylpyridinium chloride precipitation, heparin-agarose affinity chromatography, and gel filtration as described previously [41]. Tryptase activity was monitored during purification by the hydrolysis of N^{α}-benzoyl-dl-Arg-4-nitroanilide (Bz-Arg-NH-Np) [19]. Some preparations of lung tryptase were purified using immunoaffinity chromatography as described previously [12]. The concentration of the purified tryptase was determined by active site titration with 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB) in a Hitachi F-2000 fluorescence spectrophotometer (excitation $\lambda = 365$ nm, emission $\lambda = 445$ nm, 10 nm band width), and expressed as moles of active site [17].

1D and 2D gel electrophoresis

SDS/PAGE (1D) was performed on 10% polyacrylamide slab gels on a mini-Protean II Cell (Bio-Rad, Hemel Hempstead). Procedures for 2D gel electrophoresis on this apparatus were modified from the method reported previously [20,42]. Isoelectric focusing gels were prepared in glass tubes from a degassed solution of 8.5 m urea, 4% (w/v) acrylamide/bisacrylamide (Bio-Rad), 2% (v/v) Chaps detergent, 3.2% (w/v) Biolyte 5/7, 0.8% (w/v) Biolyte 3/7 (both ampholines from Bio-Rad). Mast cell preparations which had been sonicated for 5 min or purified tryptase were incubated in urea sample buffer [9 m urea, 4% (w/v) Biolyte 3/10, 2% (v/v) Chaps, 6.5 mm dithiothreitol, pH 3.5] for 45 min at 20 °C, and clarified by centrifugation at 42 000 ***g*** for 60 min at 20 °C, before loading onto gels. The anolyte solution was 20 mm l-glutamic acid, and 50 mm l-arginine was the catholyte solution. Electrophoresis was conducted at a constant voltage of 500 V for 10 min and then at 750 V for 3.5 h. The pH gradient established in the gel was measured using a surface pH electrode (Unicam) placed at 5 mm intervals along the length of the gels. The gels were extruded from the tubes into an equilibration buffer [62.5 mm Tris/HCl, 10% (v/v) glycerol, 3 mm dithiothreitol, 2.3% (w/v) SDS, pH 6.8] and incubated for 10 min at 20 °C. The gels were placed on 10% (w/v) polyacrylamide slab gels, and electrophoresis in the second dimension was performed at a constant voltage of 175–200 V for 35–40 min. Molecular mass standards employed were hen egg white lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31 kDa), hen egg white ovalbumin (45 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase b (97.4 kDa; all from Bio-Rad). Gels were stained with silver stain (Bio-Rad) or were subjected to blotting.

Western blotting

Western blotting was carried out in a wet transfer system and after blocking with 1.0% (w/v) skimmed milk power or 2% (w/v) BSA in Tris-buffered saline (TBS; 500 mm NaCl, 20 mm Tris/HCl, pH 7.5) for 1 h, blots were probed with the antitryptase monoclonal antibody AA5 (produced as previously

described [19]) and followed by treatment with biotinylated rabbit anti-mouse IgG (Dako, High Wycombe, UK) and avidin–biotin peroxidase complex (Dako). Color was developed with diaminobenzidine and hydrogen peroxide.

Lectin binding studies

Following the standard blotting procedure, filters were heated and blocked at 56 °C for 30 min in 100 mL TBS containing 2% (w/v) BSA, then 0.2 mL Tween 20 was added and incubation continued for 1 h. Horseradish peroxidase-conjugated lectins concanavalin A (Con A), wheat germ agglutinin (WGA), and phytohemagglutinin-L (PHA-L; all from Sigma), were incubated with the filters for 45 min at a concentration of 5 $\mu g \cdot mL^{-1}$, and the blots washed and incubated with diaminobenzidine and hydrogen peroxide. A combination of the biotinylated lectins *Sambucus nigra* agglutinin (SNA; 10 $\mu g \cdot mL^{-1}$) and *Maackia amurensis* agglutinin (MAA; 10 $\mu g \cdot mL^{-1}$; both from Boehringer Mannheim) was incubated with filter for 45 min, followed by incubation with avidin-biotin peroxidase complex and color development allowed to proceed with diaminobenzidine.

Deglycosylation

Oligosaccharides were removed from unseparated mast cell proteins by treatment with PNGase F or neuraminidase (both from Boehringer Mannheim) as previously described [20]. Briefly, mast cell preparations (approximately 10^6 cells) were heated at 95 °C for 5 min in 100 µL 3 mm EDTA, 0.2% (w/v) SDS and 2 mm phenylmethanesulfonyl fluoride, 10 mm Tris/HCl, pH 7.0. Samples were cooled and divided into two 50 µL aliquots. To one was added 6 U PNGase F or 0.3 U neuraminidase in 60 µL digestion buffer (3 mm dithiothreitol, 2% Chaps, 2 mm phenylmethanesulfonyl fluoride, 100 $\mu g \cdot mL^{-1}$ hen trypsin inhibitor (type III; Sigma) 5 mm EDTA, 10 mm Tris/HCl, pH 8.5), and to the other was added 60 µL digestion buffer alone. Samples were incubated for 8 h at 37 °C, after which proteins were precipitated with 1 mL of 10% (v/v) trichloroacetic acid, washed with 1% (v/v) trichloroacetic acid, redissolved in Tris/HCl, heated at 95 °C for 5 min, and analyzed on 1D or 2D electrophoresis gels.

Substrate profile

The chromogenic substrates MeOCO-Nle-Gly-Arg-NH-Np, tosyl-Gly-Pro-Arg-NH-Np and tosyl-Gly-Pro-Lys-NH-Np were purchased from Boehringer; <Glu-Gly-Arg-NH-Np, <Glu-Pro-Arg-NH-Np, Z-d-Arg-Gly-Arg-NH-Np, d-Phe-Pip-Arg-NH-Np, d-Val-Leu-Arg-NH-Np, d-Pro-Phe-Arg-NH-Np and MeO-Suc-Arg-Pro-Tyr-NH-Np from Chromogenix (Sweden); Bz-Arg-NH-Np and Suc-Ala-Ala-Pro-Phe-NH-Np from Sigma. Substrates were dissolved in dimethyl sulfoxide to 88.8 mm, and diluted in assay buffer (1.0 $mg \cdot mL^{-1}$ BSA, 1.0 m glycerol, 0.10 m Tris/HCl, pH 8.0) to 0.555 mm. As 90 µL of assay mixture was added to 10 µL sample, the final substrate concentration was 0.50 mm. Samples of tryptase for assay were adjusted to 1.0 m NaCl, 0.10 mm Tris/HCl (pH 8.0), to produce an ionic strength of approximately 0.15 m in the final reaction mixture. Assays were conducted in triplicate in microtiter plates at room temperature [43].

Enzyme kinetics

Assays were conducted as for the substrate profile except that the substrate concentration was varied from 0.025 mm to 4.0 mm and the concentration of dimethylsulfoxide was kept constant at 4.5% (v/v). Assignment to kinetic type was based on plots of v vs. [S] and [S]/v vs. [S] (Hanes' plot), and on

comparison of different mathematical models to obtain the best fit. Kinetic constants for combinations of enzyme and substrate that displayed Michaelis–Menten kinetics, positive cooperativity, or negative cooperativity were determined by a direct fit of nontransformed data to either the Michaelis–Menten equation or the Hill equation using the curve-fit function of fig.p software (version 2.7), while for those that followed simple substrate inhibition, the constants were determined by a binomial curve fit to the Hanes' plot.

Mathematical modeling

Modeling was carried out on a spreadsheet (quattro pro). Values of v and [S]/v were calculated for 100 different values of [S] for each combination of input parameters of K_m, k_{cat} and enzyme concentration. The values for the concentration of each isoform were adjusted so that the total amount of enzyme was the same for each scenario. Residuals from curve fits were calculated with the spss statistical package.

pH profile

The activity of purified tryptases from lung and skin was determined with 0.5 mm <Glu-Pro-Arg-NH-Np in buffers formulated to maintain a constant ionic strength (I = 0.15) [44]. These contained either 50 mm acetic acid, 50 mm Aces, 100 mm Tris, 50 mm NaCl (pH 4.0–6.5) or 100 mm Aces, 52 mm Tris, 52 mm 2-amino-2-methylpropanol, 50 mm NaCl (pH 6.0–10.5). Each reaction mixture also contained 0.9 $mg \cdot mL^{-1}$ BSA and 0.6% (v/v) dimethylsulfoxide. Tryptase samples were formulated in 0.12 m NaCl, 50 mm Tris/HCl, pH 7.6 with or without the addition of heparin. Assays were conducted in triplicate in microtiter plates at 20 °C [43].

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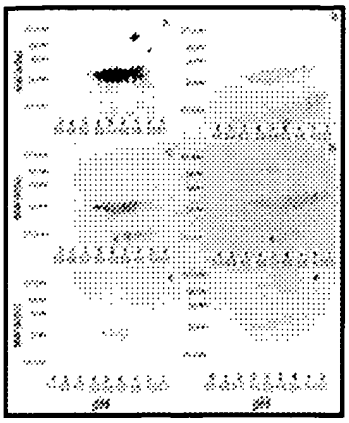
Lung mast cell tryptase

Two-dimensional gel electrophoresis of lung mast cell lysates revealed numerous silver-stained proteins ranging in molecular mass from approximately 16–120 kDa within the selected pH range of 5.0–6.7 (Fig. 1A). The patterns obtained with 10 different preparations of lung tissues were of broadly similar appearance. There was a series of intensely stained bands with pI of 5.1–6.3 and molecular masses of 30–37 kDa, which were identified as tryptase by Western blotting with monoclonal antibody AA5 (Fig. 1B).

Fig. 1. Two-dimensional gel electrophoresis of lysates of purified lung mast cells.(A) Silver stained 2D gel of sample LMC7. **(B)** Western blot of same sample probed with the anti-tryptase Ig AA5. (C–E) Western blots of preparations from other donors (LMC1, 8 and 10), and **(F)** a preparation of purified lung tryptase (LT1), all probed with AA5.



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Some 9–12 diffuse bands of lung tryptase were detected and the most dense fell within the pH range 5.6–5.9, and had molecular masses of 30–35 kDa. The molecular mass of the diffuse bands increased with declining pI from 6.2 to 5.1. The greatest range of molecular mass was found for forms of tryptase with isoelectric points between 5.1 and 5.6. The staining pattern obtained for tryptase was very consistent when the same preparation of mast cell lysate was analyzed on different occasions (not illustrated). However, there were differences in the range of both molecular mass and isoelectric point of tryptase from different lysates. The greatest variability between samples was found within the pI range of 5.1 and 5.6. In some lysates of purified lung mast cells, tryptase bands were absent within the molecular mass range of 30–37 kDa and the pI range of 5.1–5.6 (Fig. 1E). The size and charge range calculated for these bands is shown for lysates of 10 different lung mast cell preparations examined (Table 1).

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Table 1. Mean lower and upper values for molecular weight (kDa) for isoelectric point determined for immunoreactive tryptase monomers, dimers, trimers, tetramers and degradation products in Western blots of the lysates of purified lung or skin mast cells and of preparations of tryptase purified from lung or skin tissues. The SEMs are indicated in parenthesis below the mean value.

In four out of the 10 lung mast cell lysates prepared, there were bands with molecular mass of some 12–25 kDa which reacted with AA5 (Fig. 1B-D; Table 1). These may represent degradation products of tryptase. Additional bands of 62–76, 88–98 and 120–135 kDa which might represent dimers, trimers and tetramers of tryptase were observed in five of the 10 preparations. Monomeric tryptase was the major form present, and was represented by bands which were much larger and more intense than those for dimeric tryptase. There was in all cases a corresponding reduction in band size and staining intensity with increasing degree of oligomerization, so that in some cases the multimeric forms were difficult to discern.

Purified preparations of lung tryptase exhibited bands corresponding to the dominant monomeric tryptase bands seen in mast cell lysates, except that they appeared to be less diffuse. Purified tryptase had a similar range of molecular masses and pI values as did the mast cell lysates, which suggests that the purified tryptase was representative of the unfractionated tryptase within intact mast cells (Fig. 1F; Table 1). This was a consistent finding with purified lung tryptase, whether isolated by heparin agarose and gel filtration ($n = 4$) or by heparin agarose and immunoaffinity chromatography ($n = 1$). The degradation products observed in certain of the lung mast cell lysates were not detected in any of the five purified lung tryptase preparations, although the multimeric forms were observed.

Skin mast cell tryptase

Lysates of purified skin mast cells analyzed by 2D gel electrophoresis with silver staining showed a pattern of bands reminiscent of that for lung mast cells over a similar range of pI and molecular mass. Tryptase monomers identified in the blots of the skin mast cell lysates exhibited a wider range of molecular mass than lung mast cell lysates (Fig. 2; Table 1). Although the lowest molecular mass forms of the tryptase monomers were of similar size in both tissues, the highest molecular mass forms were of greater size in skin mast cell lysates than the lung lysates ($P < 0.01$, Mann–Whitney U-test) and there was a mean difference of 3 kDa in size between two tissues. Dense bands in the acidic region of gels (pH 5.1–5.6) were more common in skin samples than in lung samples. Dimers, trimers and tetramers were also observed. Degradation products were seen more frequently in lysates of purified skin mast cells (eight out of 12) compared with lung mast cells (four out of 10). Tryptase patterns in the lysates were similar to those observed in purified preparations of skin tryptase including the presence of breakdown products.

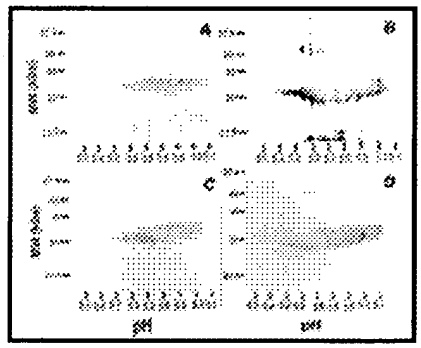

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Fig. 2. Two-dimensional gel electrophoresis of lysates of purified skin mast cells. Western blots probed with anti-tryptase Ig AA5 for (A–C) mast cells purified from skin tissue (SMC1, 6 and 10), and (D) a preparation of purified skin tryptase (ST2).

Identification of glycoproteins

The lectins SNA and MAA, which bind specifically to sialic acids, bound strongly to tryptase bands identified in blots of lysates of both lung (Fig. 3B) and skin mast cells (results not shown), providing evidence that tryptase is sialylated. In addition, there were certain proteins other than tryptase which were also stained positively with SNA/MAA, which had a molecular mass of 60–70 kDa and appeared to be present in greater amounts in the skin lysates than in lung lysates. Con A, a lectin which binds to

mannose of asparagine-linked oligosaccharides [45,46], also bound to tryptase from both lung (Fig. 3C) and skin lysates (results not shown). WGA, a lectin which binds specifically to *N*-acetylglucosamine and to a certain extent to sialic acids as well [47,48], also bound to tryptase (Fig. 3D). All tryptase bands recognized by AA5 antibody bound to each of the lectins. There seemed to be stronger SNA/MAA-binding, but weaker WGA-binding, to skin than to lung tryptase, though a similar difference was not observed in the intensity of staining with AA5 antibody. The lectin PHA-L, a lectin which is selective for complex-type structures which are at least triantennate [49,50], did not bind to any of the separated lung or skin mast cell preparations, so the complex-type carbohydrate in tryptase is more likely to be mono-antennate or bi-antennate.

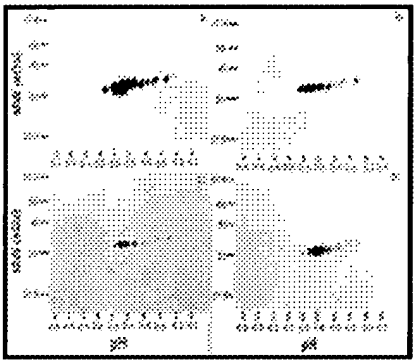

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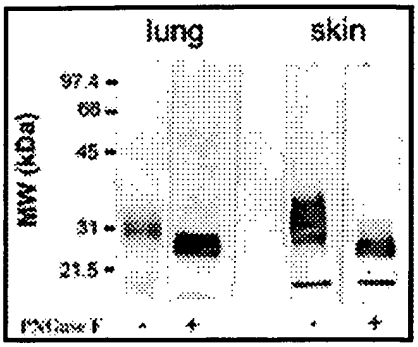
Fig. 3. Lectin binding to lung mast cell tryptase. Matching blots of a lysate of lung mast cells (sample LMC2) subjected to 2D gel electrophoresis were probed with (A) tryptase-specific antibody AA5 **(B)** lectins SNA and MAA **(C)** Con A and **(D)** WGA.

Deglycosylation of tryptase

Incubation of lung or skin mast cell lysates with PNGase F to remove asparagine-linked carbohydrates resulted in a reduction in the molecular mass of tryptase on blots and a sharpening of the bands (Fig. 4). There was a greater reduction in the molecular mass of skin tryptase (from 29–38 to 26–29 kDa for the monomers) than for lung tryptase (30–34 to 26–30 kDa). The molecular mass of purified lung tryptase was also reduced following treatment with PNGase F (Fig. 5), though to a lesser extent (from 30–36 to 30–33 kDa on blots probed with AA5) than with tryptase in the lung mast cell lysates. Lectin binding studies with SNA/MAA indicated that carbohydrate chains (and sialic acid residues) had to a large extent been removed by treatment with PNGase F.

Fig. 4. Effect of PNGase F on tryptase molecular mass. Lysates of purified mast cells from lung or skin were incubated in the absence (–) or presence (+) of PNGase F. Samples were analyzed by SDS/PAGE and Western blotting with antibody AA5.



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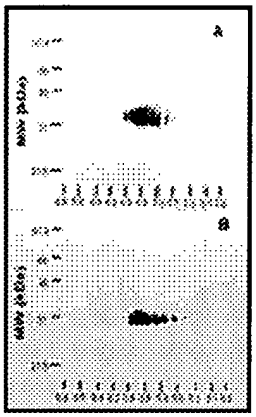

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Fig. 5. The effect of deglycosylation on the size, charge and lectin-binding properties of tryptase, as revealed by 2D gel electrophoresis. Blots of purified lung tryptase, which had been incubated in the absence (A) or presence (B) of PNGase F, were probed with AA5 antibody.

In the 2D gel analysis, Western blots of tryptase incubated with PNGase F under denaturing conditions indicated that the reduction in molecular size affected bands of different charge differently (Fig. 5). Overall the molecular size of monomeric lung tryptase was reduced from 30–38 to 27–34 kDa. The greatest reduction in size was observed for tryptase forms in the pH range 5.2–5.6, while the dominant dense bands with pI of 5.6–5.9 showed only a marginal reduction in molecular weight. PNGase F treatment was also associated with a narrowing in the range of pI values from 5.2–6.2 to 5.4–6.0. Where present, the size of multimeric forms of tryptase was also reduced, with the greatest reductions again in the bands in the acidic range. Incubation of tryptase with PNGase F markedly reduced the ability of the lectins SNA/MAA to bind to blots, which indicates that most sialic acid residues had been removed with the N-linked carbohydrates (results not shown).

Treatment of tryptase with neuraminidase resulted in a reduction in molecular mass from 28–43 to 26–38 kDa (Fig. 6). Neuraminidase also induced a narrowing in the pI range from 5.2–6.3 to 5.5–6.1, and

fewer distinct bands were observed in the pH 5.6–6.1 region.

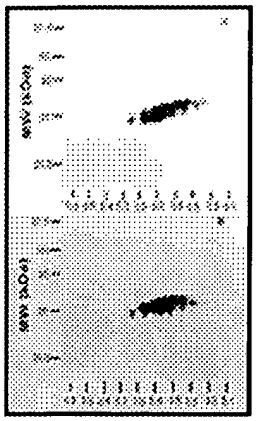

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Fig. 6. The effect of desialylation on the size, charge and lectin-binding properties of tryptase, as revealed by 2D gel electrophoresis. Blots of purified lung tryptase, which had been incubated in the absence (A) or presence (B) of neuraminidase, were probed with AA5 antibody.

Substrate profile

The action of four separate isolates of tryptase (L1 and L2 from lung and S1 and S2 from skin) was tested on a range of substrates, each at 0.50 mm, and compared with the standard assay with the substrate Bz-Arg-NH-Np (Table 2). There were differences in activity between tryptase preparations, but the differences between the two skin isolates were greater than those between lung and skin. This can be seen particularly with Z-d-Arg-Gly-Arg-NH-Np: the molar catalytic activity of L1 was less than a third of that of L2 while the activities of L2, S1, and S2 were all much the same. Although the values for molar catalytic activity differed between isolates, the relative order of substrate preference was virtually the same for all four preparations. Comparison of tosyl-Gly-Pro-Arg-NH-Np with tosyl-Gly-Pro-Lys-NH-Np revealed a preference of an approximately 1.5-fold for arginine over lysine at the P1 position, while comparison of <Glu-Pro-Arg-NH-Np with <Glu-Gly-Arg-NH-Np indicated a strong preference (approximately eightfold) for proline over glycine at position P2. Indeed, all four tryptase isolates favored substrates with proline at P2 over all other substrates tested, while the substrate with the 6-membered-ring analog of proline, pipecolic acid, at P2 ranked next.

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Table 2. Activity of different purified preparations of tryptase against a range of substrates. All substrates were at a concentration of 0.50 mm, except for the Bz-Arg-NH-Np standard, which was at 0.9 mm.

Kinetics

Efforts to determine the kinetic constants of the different isolates of tryptase for each of the substrates produced a range of behavior including standard Michaelis–Menten kinetics (Fig. 7A,E), substrate inhibition (Fig. 7B,F), positive cooperativity (Fig. 7C,G), and negative cooperativity (Fig. 7D,H).

The results are summarized in Table 3. Discrepancies between the data and the standard Michaelis–Menten model were not as obvious on v vs. [S] plots (Fig. 7C,D) as they were on the Hanes' plot (Fig. 7G,H) or in plots of the residuals (results not shown). Identification of the type of kinetics for a particular combination of enzyme and substrate was based on the shape of the Hanes' plot (linear for Michaelis–Menten kinetics, concave upwards for substrate inhibition and positive cooperativity, and concave downwards for negative cooperativity) and the best fit to alternative mathematical models. The decision could be subjective in a few cases; for example, although S2 gave a reasonable fit to the substrate inhibition model with Z-d-Arg-Gly-Arg-NH-Np, the estimated value of K' was much higher than the range of [S] used, so that for practical purposes, the enzyme was deemed to obey Michaelis–Menten kinetics. Also, although Hill coefficients greater than 1.2 were usually accompanied by clear sigmoidal behavior at low substrate concentrations, at other times were not, e.g. with all tryptase isolates in the presence of Z-d-Arg-Gly-Arg-NH-Np. In these cases it appeared the computational algorithm was driven by the flattening or decrease of activity at high substrate concentration rather than by any sigmoidal behavior at low substrate concentration.

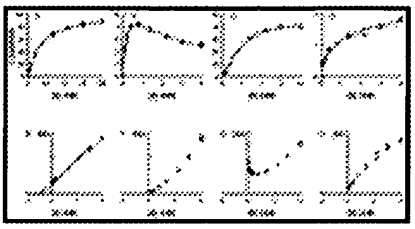

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Fig. 7. Variety of kinetic patterns observed with tryptase. Results are plotted as rate of reaction (v) vs. substrate concentration ([S]) (A–D) and as [S]/v vs. [S] (the Hanes plot) (E–H). Examples of kinetic types are Michaelis–Menten kinetics (A,E) obtained with <Glu-Pro-Arg-NH-Np and tryptase S1, substrate inhibition (B,F) obtained with Z-d-Arg-Gly-Arg-NH-Np and tryptase S1, positive cooperativity (C and G) obtained with MeOCO-Nle-Gly-Arg-NH-Np and tryptase S1, and negative cooperativity (D,H) obtained with d-Pro-Phe-Arg-NH-Np and tryptase L1. Solid curves are those fit to the corresponding mathematical model. Dotted curves (C,D) are those fit to the Michaelis–Menten equation with the same data.

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Table 3. Kinetic constants for combinations of enzyme and substrate tested.

The behavior differed from substrate to substrate and from isolate to isolate (Table 3). For example, although consistent $K_{0.5}$-values were obtained for the four tryptase preparations with tosyl-Gly-Pro-Lys-NH-Np and d-Phe-Pip-Arg-NH-Np, there was a 16-fold difference in K_m values for Bz-Arg-NH-Np between isolates L1 and S1. Different kinetics between isolates towards the same substrate were obtained for d-Val-Leu-Arg-NH-Np, Bz-Arg-NH-Np, and d-Pro-Phe-Arg-NH-Np. The disparity in activity between isolates from the same tissue was often greater than that between tissues.

Mathematical modeling

The possibility that the variety of kinetic patterns observed was the consequence of a heterogeneous

population of tryptase isoforms, each with its own values of K_m and k_{cat}, was examined by mathematical modeling. In this model, each isoform was assumed to be independent of all other isoforms and to obey simple Michaelis–Menten kinetics (Eqn 1):

$$v = \frac{k_1 E_1 s}{s + K_{m1}} + \frac{k_2 E_2 s}{s + K_{m2}} + \frac{k_3 E_3 s}{s + K_{m3}} + \frac{k_4 E_4 s}{s + K_{m4}} + \frac{k_5 E_5 s}{s + K_{m5}}$$

$$+ \frac{k_6 E_6 s}{s + K_{m6}} \qquad (1)$$

A range of values were chosen for k_i, E_i and K_{mi}, and v and s/v were calculated. If all forms had the same K_m but different concentrations or k_{cat} values, then the Hanes' plot was linear ($r^2 = 1.0000$), yielding the input value of K_m as K_m and a weighted average of the input values of k_{cat} as the computed value of k_{cat} (case 1 of Fig. 8A). If each form had a different value of K_m, however, although the Hanes' plot might appear linear (e.g. case 2 of Fig. 8A), r^2 was not unity and a plot of residuals indicated that the Hanes' plot was a curve concave downwards (Fig. 8B). This curvature could be made more readily apparent by altering $[E_i]$ values as well as K_{mi} values (case 4 of Fig. 8A). In all cases modeled, the curve was concave downwards, never upwards as most deviations from linearity were with tryptase. This shape of curve for multiple forms of an enzyme is in agreement with that previously reported for a binary mixture [51 and references cited therein].

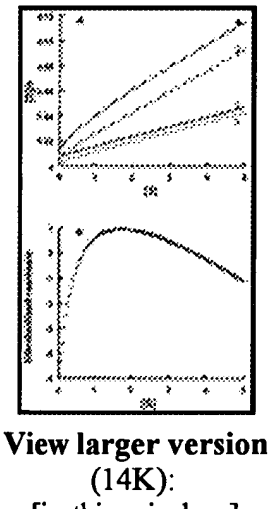

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Fig. 8. Mathematical modeling of the behavior of a mixture of isoforms of an enzyme.(A) Hanes plot of a theoretical mixture of 5 isoforms of an enzyme for the following cases: (1) [E1] = [E2] = [E3] = [E4] =[E5]; $K_{m1} = K_{m2} = K_{m3} = K_{m4} = K_{m5}$; $k_{cat1} < k_{cat2} < k_{cat3} < k_{cat4} < k_{cat5}$; (2) [E1] = [E2] = [E3] = [E4] = [E5]; $K_{m1} > K_{m2} > K_{m3} > K_{m4} > K_{m5}$; $k_{cat1} = k_{cat2} = k_{cat3} = k_{cat4} = k_{cat5}$; (3) [E1] = [E2] = [E3] = [E4] = [E5]; $K_{m1} > K_{m2} > K_{m3} > K_{m4} > K_{m5}$; $k_{cat1} < k_{cat2} < k_{cat3} < k_{cat4} < k_{cat5}$; (4) [E1] > [E2] > [E3] > [E4] > [E5]; $K_{m1} > K_{m2} > K_{m3} > K_{m4} > K_{m5}$; $k_{cat1} = k_{cat2} = k_{cat3} = k_{cat4} = k_{cat5}$. **(B)** plot of the standardized residuals for a linear regression fit to the data generated by case 2 above.

In order to determine whether the curve of the Hanes' plot of this model could ever be concave upwards, the general case was considered. For n independent forms of an enzyme, each with its own values of K_m,

k_{cat} and concentration and obeying Michaelis–Menten kinetics, the Hanes' plot takes the form

$$\frac{s}{v} = \frac{s^n + a_{n-1}s^{n-1} + a_{n-2}s^{n-2} + \cdots + a_2s^2 + a_1s + a_0}{b_{n-1}s^{n-1} + b_{n-2}s^{n-2} + \cdots + b_2s^2 + b_1s + b_0} \quad (2)$$

where a_i and b_i are derived from the input parameters.

At s = 0,

$$\frac{s}{v} = \frac{a_0}{b_0}$$

where $a_0 = K_{m1}K_{m2}K_{m3} \ldots K_{mn}$ and $b_0 = k_1E_1 (K_{m2}K_{m3} \ldots K_{mn}) + k_2E_2(K_{m1}K_{m3} \ldots K_{mn}) + \ldots + k_iE_i (K_{m1}K_{m3} \ldots K_{mi-1}K_{mi+1} \ldots K_{mn}) + \ldots + k_nE_n(K_{m1}K_{m3} \ldots K_{mn-1})$

This simplifies to

$$\frac{s}{v} = \frac{1}{\frac{k_1E_1}{K_{m1}} + \frac{k_2E_2}{K_{m2}} + \cdots + \frac{k_nE_n}{K_{mn}}} \quad (3)$$

At very large values of s, the Hanes' equation approaches

$$\frac{s}{v} = \frac{s^n + a_{n-1}s^{n-1}}{b_{n-1}s^{n-1}}$$

$$= \frac{s}{b_{n-1}} + \frac{a_{n-1}}{b_{n-1}} \quad (4)$$

where $a_{n-1} = \Sigma K_{mi}$ and $b_{n-1} = \Sigma k_iE_i$.

Thus, the curve for the Hanes plot asymptotically approaches a line which has as its slope 1/(sum of the V_{max} values for each isoform) and a y-intercept which can be rewritten

$$\frac{s}{v} = \frac{1}{\frac{k_1E_1}{\sum K_{mi}} + \frac{k_2E_2}{\sum K_{mi}} + \cdots + \frac{k_nE_n}{\sum K_{mi}}} \quad (5)$$

The Hanes curve can only ever be concave upwards if its value at $x = 0$ is greater than the y-intercept of

the asymptote. Comparison of the terms in the denominators of Eqns 3 and 5 shows that for positive values of K_{mi}, the terms of the denominator of Eqn 5 will always be less than the corresponding terms in Eqn 3. As the number of terms is the same for both equations, the value of the y-intercept for the asymptote will always be greater than the value of the Hanes curve at $x = 0$. Therefore, for real enzymes, which can only have positive values of K_m, the presence of a multiplicity of isoforms, each obeying Michaelis–Menten kinetics, can not mimic the behavior of a single form displaying sigmoidal kinetics or substrate inhibition.

However, a multiplicity of isoforms could account for the behavior of tryptase L1 with d-Pro-Phe-Arg-NH-Np (Fig. 7D,H). The data for this substrate-isolate pair did fit to a two-enzyme model, but the iteration converged on an unrealistically high value for K_m for the second enzyme (42 000 mm). Alternatively, if the second enzyme was treated as being in the linear range (as was observed with <Glu-Gly-Arg-NH-Np), a very good fit was obtained, with K_m and V_{max} values of 0.20 mm and 1.14 s^{-1}, respectively, for the first enzyme, and a V_{max}/K_m ratio of 187 $s^{-1} \cdot m^{-1}$ for the second enzyme. (V_{max}, rather than k_{cat}, values pertain in this case, as the model does not resolve the relative proportions of the two enzymes.)

pH profile

The activity of lung (L1) and skin (S1) tryptase over a pH range of 4.0–10.5 was determined using <Glu-Pro-Arg-NH-Np as substrate, both in the presence (100 $\mu g \cdot mL^{-1}$) and absence of heparin (molecular mass range of 13–15 kDa) (Fig. 9). There was no apparent difference between the two isolates. For both isolates, heparin had little effect, except at pH 10.0, where it offered some degree of stabilization. In the presence of heparin at this pH, the progress curves showed an exponential loss of activity with a half-life of 3.3 and 3.8 min for lung and skin tryptases, respectively. In the absence of heparin at this pH, activity was almost completely lost during the interval between addition of substrate and the first reading (1 min). At pH values ≤ 9.5, all progress curves were linear throughout the course of the assay (14 min), whether or not heparin was present.

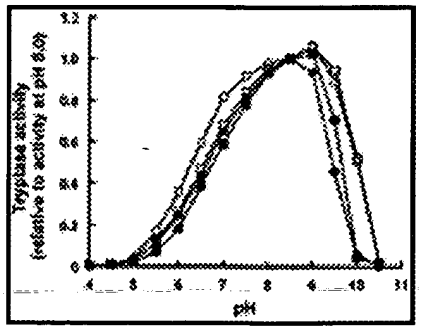

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Fig. 9. pH profile of human skin and lung tryptase in the presence and absence of heparin. (■) skin tryptase, no heparin (□) skin tryptase + 100 $\mu g \cdot mL^{-1}$ heparin (•) lung tryptase, no heparin (○) lung tryptase + 100 $\mu g \cdot mL^{-1}$ heparin.

Discussion

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We have found human tryptase to be highly heterogeneous in size, charge and activity, and that differences are related not just to the tissue source, but also to the individual from whom cells were collected or from whom the enzyme was purified. Lectin-binding and glycosidase studies have shown that differences in glycosylation contribute significantly to this microheterogeneity in size and charge, but the present evidence does not rule out a possible contribution from multiple alleles or genes. The chemical basis for the marked differences in activity and kinetic behavior was not ascertained, but mathematical modeling ruled out the possibility that such diversity could arise through a mixture of isoforms obeying hyperbolic kinetics, but with differing values of K_m and k_{cat}.

The results of our 2D gel studies are in agreement with and extend the findings of Benyon *et al.* [20], who examined lysates of skin mast cells. We also observed a similar degree of microheterogeneity in mast cells isolated from lung and in tryptase purified from both sources. This technique gave a clear separation of different forms of tryptase on the basis of isoelectric point (the first dimension), but not on the basis of size. Rather, a gradation was seen between the lower and higher molecular mass forms of tryptase of similar isoelectric point. A situation analogous to that observed with one dimensional gel electrophoresis, in which two [16,17] or more [18] distinct forms differing in size by 2–4 kDa were resolved, was not seen using the more sensitive procedure. There was some association between pI and molecular mass. With declining pI, the size of tryptase monomers showed a gradual increase, consistent with a correlation between the degree of sialylation and size/number of N-linked oligosaccharides.

The results of the lectin-binding studies together with the effects of treatment with neuraminidase and PNGase F indicate that much of the heterogeneity is due to differences in glycosylation. All spots which reacted with the antitryptase antibody AA5 also reacted with one or more lectins, with the possible exception of the lowest molecular mass spots for both lung tryptase (29 ± 1.5 kDa) and skin tryptase (29 ± 2.4 kDa), which had masses similar to those calculated from the amino acid sequence (approximately 27.5 kDa) [26-28]. However, these low molecular mass forms appear to be present in only small quantities in the preparations, suggesting that most tryptase is glycosylated, utilizing either one or both potential N-glycosylation sites. Tryptases α, βI, and an allelic variant of βII have two such sites [26,28,38], while βIII and the reference sequence for βII have only one [27,28]. The site common to all tryptases (Asn194) occurs in a consensus Asn-X-Thr sequence, while the additional site (Asn99) is present in an Asn-X-Ser sequence. Efficiency of glycosylation at any particular site is dependent on a number of possible factors [52], but the identity of the third amino acid in the consensus sequence is one of them. Sequences with serine in the third position tend to be less efficiently utilized than those with threonine. Site-directed mutagenesis studies with antithrombin III showed that substitution of the native Ser at one site with Thr improved the extent of glycosylation and, conversely, replacement of native Thr with a Ser decreased the efficiency of glycosylation at most, but not all, of the other sites [52]. Whether Asn99 is less efficiently used than Asn194 would require further investigation, but partial glycosylation

at this site could account for some of the heterogeneity seen.

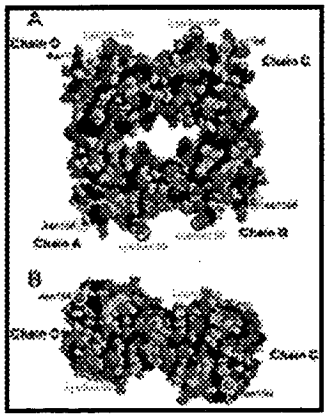
Treatments with PNGase F reduced the range of both molecular mass and pI values, but did not reduce tryptase to a single spot on 2D blots, probably because the deglycosylation reaction did not go to completion, as indicated by the continued reaction with lectin. A reduction in the molecular mass following PNGase F treatment has been reported previously with skin (31–36 to 30 kDa) [20], and pituitary tryptase (32.4–36.3 to 32.4 kDa) [18]. It is not clear why a decrease in the size of lung tryptase with PNGase F treatment has not been observed by other workers [16,18]. Treatment of tryptase with neuraminidase, which removes sialic acid residues, resulted in a smaller, but significant, reduction in molecular mass. As the sialic acid residue has a formula mass of 291 Da, these results would suggest extensive sialylation of the tryptase molecule.

Lectin binding studies provide additional evidence for extensive sialylation as indicated by the strong reaction with SNA/MAA. Mannose is also present in most isoforms of tryptase as shown by reaction with Con A. Although Con A binds strongly to high-mannose type of oligosaccharide, it also binds to relatively small complex-type structures with a low degree of branching [45,46]. Lectin histochemical studies have indicated that the high-mannose type was not a major class in mast cell granules because of the lack of an effect of α-mannosidase on the binding of Con A [53]. This would suggest that tryptase, the major granular constituent, is not a high mannose type of glycoprotein, and that positive staining achieved with Con A may reflect the presence of mannose only in the backbone of complex-type oligosaccharides with a low degree of branching. The failure of PHA-L to bind to tryptase provides further evidence for a low degree of branching [49,50]. The presence of complex-type carbohydrates is supported by the reaction with WGA, which can bind *N*-acetyl-d-glucosamine residues, but can also bind some sialyl residues [47,48].

The present evidence does not rule out a possible contribution to the observed heterogeneity from multiple genes or alleles. On the basis of the two-locus model proposed by Soto and coworkers [34], allelic variation at the first locus between α and βI, and at the second locus between βII and βIII, would give rise to nine possible genotypes (αα$^{\beta II}$$^{\beta II}$, αα$^{\beta II}$$^{\beta III}$, αα$^{\beta III}$$^{\beta III}$, α$^{\beta I}$$^{\beta II}$$^{\beta II}$, α$^{\beta I}$$^{\beta II}$$^{\beta III}$, α$^{\beta I}$$^{\beta III}$$^{\beta III}$, $^{\beta I}$$^{\beta I}$$^{\beta II}$$^{\beta II}$, $^{\beta I}$$^{\beta I}$$^{\beta II}$$^{\beta III}$, $^{\beta I}$$^{\beta I}$$^{\beta III}$$^{\beta III}$). Additional complexity is generated by the existence of numerous SNPs for both tryptase loci, including six amino acid variants and two frameshift mutants for βII-tryptase and six amino acid variants for α-tryptase [38]. The antitryptase antibody used in this study reacts equally well with both α- and βII-tryptase [54], and in view of the very high homology between the β-tryptases, would be expected to cross-react readily with βI and βIII as well, and probably with most SNPs. Therefore, any or all of these genetic variants could be contributing to the observed heterogeneity. However, a comparison of immunoassays, which differ in their affinity for α-tryptase but have similar affinities for β-tryptase, suggested that α-tryptase is constitutively secreted whilst β-tryptase is stored in the granules of developing mast cells [36]. In support of this scheme is the delineation of a possible mechanism for sorting the α- and β-proenzymes to different post-Golgi pathways [37]. If this were indeed the case, and α-tryptase made a negligible contribution to the observed heterogeneity, examination of the above genotypes indicates that there would still be ample scope for a genetic contribution to the microheterogeneity within any particular sample and also to the diversity seen

between different samples.

The reported crystal structure of this enzyme [23] does not shed any light on the degree or nature of its glycosylation as the oligosaccharide chains were not seen, presumably because the heterogeneity in carbohydrate structure was 'seen' as disorder. However, examination of the crystal structure (ref 1A0L) through the website http://oca.ebi. ac.uk and firstglance software showed the potential N-glycosylation sites Asn194 (Asn204 by chymotrypsinogen numbering) and Asn99 (Lys112 in the structure of Pereira *et al.*) are exposed on the surface of the enzyme along the outer edge of the ring formed by the tetramer (Fig. 10) where they might be expected to be readily accessible to oligosaccharide transfer from dolichol pyrophosphate. These putative glycosylation sites are well away from the central pore containing the active sites, so are unlikely to cause steric hindrance with any substrate. They are also away from the putative heparin-binding site, a region of positive surface charge extending along the left- and right-hand sides of the ring in Fig. 10A[23]. This region is comprised of five histidines, nine lysines, and four arginines in each subunit. The pH profile data suggest that as the pH increases, there is still sufficient protonation of the lysines at pH 10, along with the fully protonated arginines, to interact with the heparin to delay inactivation of the enzyme, but by pH 10.5, too many of the lysine residues have become deprotonated for heparin to afford any stability.



View larger version (65K): [in this window] [in a new window]

Fig. 10. Three-dimensional structure of tryptase [23] indicating positions of potential N-glycosylation sites. Structure viewed at http://oca.ebi. ac.uk using a netscape browser and firstglance software. **(A)** View showing tetrameric structure and the central pore containing the four active sites. (B) Orthogonal view of top of the ring structure shown in (A). Color code is black, peptide backbone; grey, nonpolar residues; pink, uncharged polar residues; red, acidic residues; and purple, basic residues. Tryptases α, $^{\beta}$I, and an allelic variant of $^{\beta}$II [38] have an asparagine at position 99, while $^{\beta}$III and the reference sequence for $^{\beta}$II have a lysine. All isoforms have an asparagine at position 194.

Although there were broad similarities in the range of pI expressed and in the patterns obtained as well as significant variation between donors, consistent differences did emerge between lung and skin tryptase. Lung tryptase exhibited a narrower range of molecular masses than did skin tryptase on 2D gels, which suggests a narrower size distribution in the associated oligosaccharides. Differences in composition of these carbohydrates were also suggested by differences in staining intensity in lectin binding studies. The lectins SNA/MAA appeared to have a higher affinity for skin tryptase than for lung tryptase. In contrast, the lectin WGA seemed to have a higher affinity for the isoforms found in lung than those in skin. This may indicate that tryptase in skin mast cells may have higher degree of

sialylation whilst tryptase in lung mast cells may have more terminal N-acetylglucosamine residues. These differences in physicochemical properties between tryptase from different anatomical sites could reflect important differences in function, such as turnover, targeting, and activity. The nature of the factors controlling post-translational modification remains to be elucidated, but are likely to be affected by local environmental conditions and by mast cell phenotype. Disease state might also affect these processes with important implications for the pathogenesis of allergic diseases.

Other differences observed on 2D gels between the lysates of lung and skin mast cells include the relative abundance of degraded and oligomeric forms of tryptase. Although breakdown products of tryptase were observed in preparations from both sources of tissue investigated, they were detected more frequently in skin preparations (eight out of 12 lysates) than in lung preparations (four out of 10 lysates), which suggests that either skin tryptase is more easily degraded or skin mast cells contain higher amount of a protease which can degrade it. As most preparations of purified tryptase did not contain any breakdown products, it is unlikely that these spots are the result of autodigestion. It is perhaps relevant that lysates of mast cells isolated from skin contain tenfold higher levels of chymotryptic activity than do purified lung mast cells [55]. One very likely explanation for the appearance of dimeric, trimeric and tetrameric forms of tryptase is that sulfydryl groups reduced during sample preparation are re-oxidized during electrophoresis in the first dimension to form intersubunit disulfide bonds. However, when all samples were subjected to the same conditions, it is not clear why such reoxidation would occur more readily and to a greater extent in lysates of skin mast cells than in those of lung.

Previous comparisons of the activity of skin and lung tryptase appeared to have examined only one preparation of each for any given substrate [21,25], with one group finding marked differences between the two [21], the other finding negligible differences [25]. By examining more than one preparation of each, we have found that differences between separate isolates from the same tissue can be greater than those between isolates from different tissues. Not only did we find differences in relative activity and in kinetic constants, e.g. a 16-fold difference in K_m values for Bz-Arg-NH-Np between isolates L1 and S1, but we also found different kinetics between isolates towards the same substrate. The variety of kinetic behavior was somewhat surprising, but not without precedent. Substrate inhibition, which was observed for Z-d-Arg-Gly-Arg-NH-Np, has been previously reported for Z-Trp-Arg-SBzl [21]. This behavior could perhaps be expected for Z-d-Arg-Gly-Arg-NH-Np, which could conceivably bind by either the P1 or the P3 argininyl residue to the S1 binding pocket. Although binding via the P3 Arg could result in cleavage of the substrate between residues P3 and P2, this reaction would go undetected, as the chromophore would still be covalently linked to the peptide. Sigmoidal kinetics has been previously reported, but in both studies, Michaelis–Menten kinetics were converted to sigmoidal kinetics by the addition of an effector, either increasing concentrations of $(NH_4)_2SO_4$ or KCl in the absence of heparin with Z-Gly-Pro-Arg-NH-Np as substrate [25], or histamine, which at 10 mm displayed a Hill coefficient of 1.31 with tosyl-Gly-Pro-Lys-NH-Np as substrate [56].

In conclusion, we have demonstrated differences in the microheterogeneity of tryptase from different tissues and different donors and have presented evidence that much of this microheterogeneity can be attributed to N-linked glycosylation. The differences observed in the kinetic properties of different

preparations of purified tryptase strongly suggest that this microheterogeneity has a direct bearing on the enzyme's behavior and this would have important ramifications for the understanding of pathophysiological roles of this enzyme.

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Footnotes

Enzyme: serine protease tryptase (EC 3.4.21.59).

Note: a web site is available at http://www.som.soton.ac.uk/research/rcmb/groups/mast-baso.htm

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1. Walls, A.F. (2000) The roles of neutral proteases in asthma and rhinitis. In *Asthma and Rhinitis*, 2nd edn. (Busse, W.W. & Holgate, S.T., eds), pp. 968–997. Blackwell, Boston, USA.
2. Walls, A.F. (2000) Structure and function of mast cell tryptase. In *Mast Cells and Basophils in Physiology, Pathology and Host Defence* (Marone, G., Lichtenstein, L.M. & Galli, S.J., eds), pp. 291–309. Academic Press, London, UK.
3. Tam, E.K. & Caughey, G.H. (1990) Degradation of airway neuropeptides by human lung tryptase. *Am. Rev. Respir. Cell. Mol. Biol.* **3**, 27–32.[Medline]
4. Walls, A.F., Brain, S.D., Desai, A., Jose, P.J., Hawkings, E., Church, M.K. & Williams, T.J. (1992) Human mast cell tryptase attenuates the vasodilator activity of calcitonin gene-related peptide (CGRP). *Biochem. Pharmacol.* **43**, 1243–1248.[Medline]
5. Walls, A.F., Bennett, A.F., Sueiras-Diaz, J. & Olsson, H. (1992) The kininogenase activity of human mast cell tryptase. *Biochem. Soc. Trans.* **20**, 260S.[Medline]
6. Thomas, V.A., Wheeless, C.J., Stack, M.S. & Johnson, D.A. (1998) Human mast cell tryptase fibrinogenolysis: kinetics, anticoagulation mechanism, and cell adhesion disruption. *Biochemistry* **37**, 2291–2298.[Medline]
7. Cairns, J.A. & Walls, A.F. (1996) Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. *J. Immunol.* **156**, 275–283. [Abstract]
8. Cairns, J.A. & Walls, A.F. (1997) Mast cell tryptase stimulates the synthesis of type I collagen in human lung fibroblasts. *J. Clin. Invest.* **99**, 1313–1321.[Abstract/Free Full Text]
9. He, S., Gaça, M.D.A. & Walls, A.F. (1998) A role for tryptase in the activation of human mast cells: modulation of histamine release by tryptase and inhibitors of tryptase. *J. Pharmacol. Exp. Ther.* **286**, 289–297.[Abstract/Free Full Text]
10. Compton, S.J., Cairns, J.A., Holgate, S.T. & Walls, A.F. (1998) The role of mast cell tryptase in

regulating endothelial cell proliferation, cytokine release and adhesion molecule expression. Tryptase induces expression of mRNA for IL-1β and IL-8 and stimulates the selective release of IL-8 from HUVEC. *J. Immunol.* **161**, 1939–1947.[Abstract/Free Full Text]

11. Berger, P., Perng, D.-W., Thabrew, H., Compton, S.J., Cairns, J.A., McEuen, A.R., Marthan, R., Tunon-de-Lara, J.M. & Walls, A.F. (2001) Tryptase and agonists of protease activated receptor 2 induce the proliferation of human airway smooth muscle cells. *J. Appl. Physiol.* **91**, 1372–1379. [Abstract/Free Full Text]
12. He, S., Peng, Q. & Walls, A.F. (1997) Potent induction of a neutrophil and eosinophil-rich infiltrate *in vivo* by human mast cell tryptase. Selective enhancement of eosinophil recruitment by histamine. *J. Immunol.* **159**, 6216–6225.[Abstract]
13. Berger, P., Compton, S.J., Molimard, M., Walls, A.F., N'Guyen, C., Marthan, R. & Tunon de Lara, J.M. (1999) Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. *Clin. Exp. Allergy* **29**, 804–812.[Medline]
14. Clark, J.M., Abraham, W.M., Fishman, C.E., Forteza, R., Ahmed, A., Cortes, A., Warne, R.L., Moore, W.R. & Tanaka, R.D. (1995) Tryptase inhibitors block allergen induced airway and inflammatory responses in allergic sheep. *Am. J. Resp. Crit. Care Med.* **152**, 2076–2083. [Abstract]
15. Krishna, M.T., Chauhan, A., Little, L., Sampson, K., Hawksworth, R., Mant, T., Djukanovic, R., Lee, T. & Holgate, S. (2001) Inhibition of mast cell tryptase by inhaled APC 366 attenuates allergen-induced late-phase airway obstruction in asthma. *J. Allergy Clin. Immunol.* **107**, 1039–1045.[Medline]
16. Schwartz, L.B., Lewis, R.A. & Austen, K.F. (1981) Tryptase from human pulmonary mast cells: purification and characterization. *J. Biol. Chem.* **256**, 11939–11943.[Abstract/Free Full Text]
17. Smith, T.J., Hougland, M.W. & Johnson, D.A. (1984) Human lung tryptase: purification and characterization. *J. Biol. Chem.* **259**, 11046–11051.[Abstract/Free Full Text]
18. Cromlish, J.A., Seidah, N.G., Marchinkiewicz, M., Hamelin, J., Johnson, D.A. & Chretien, M. (1987) Human pituitary tryptase: molecular forms, NH_2-terminal sequence, immunocytochemical localization, and specificity with prohormone and fluorogenic substrates. *J. Biol. Chem.* **262**, 1363–1373.[Abstract/Free Full Text]
19. Walls, A.F., Bennett, A.R., McBride, H.M., Glennie, M.J., Holgate, S.T. & Church, M.K. (1990) Production and characterization of monoclonal antibodies specific for human mast cell tryptase. *Clin. Exp. Allergy* **20**, 581–589.[Medline]
20. Benyon, R.C., Enciso, J.A. & Befus, A.D. (1993) Analysis of human skin mast cell proteins by two-dimensional gel electrophoresis. *J. Immunol.* **151**, 2699–2706.[Abstract/Free Full Text]
21. Tanaka, T., McRea, B.J., Cho, K., Cook, R., Fräki, J.E., Johnson, D.A. & Powers, J.C. (1983) Mammalian tissue trypsin-like enzymes. *J. Biol. Chem.* **258**, 13552–13557. [Abstract/Free Full Text]
22. Little, S.S. & Johnson, D.A. (1995) Human mast cell tryptase isoforms: separation and examination of substrate specificity differences. *Biochem. J.* **307**, 341–346.[Medline]
23. Pereira, P.J.B., Bergner, A., Macedo-Ribeiro, S., Huber, R., Matschiner, G., Fritz, H., Sommerhoff, C.P. & Bode, W. (1998) Human β-tryptase is a ring-like tetramer with active sites facing a central pore. *Nature* **392**, 306–311.[Medline]
24. Schwartz, L.B. (1985) Monoclonal antibodies against human mast cell tryptase demonstrate shared antigenic sites on subunits of tryptase and selective location of the enzyme to mast cells. *J. Immunol.* **134**, 526–531.[Abstract/Free Full Text]
25. Harvima, I.T., Schechter, N.M., Harvima, R.J. & Fräki, J.E. (1988) Human skin tryptase: purification, partial characterization and comparison with human lung tryptase. *Biochim. Biophys. Acta* **957**, 71–80.[Medline]
26. Miller, J.S., Westin, E.H. & Schwartz, L.B. (1989) Cloning and characterization of complementary DNA for human tryptase. *J. Clin. Invest.* **84**, 1188–1195.[Medline]
27. Miller, J.S., Moxley, G. & Schwartz, L.B. (1990) Cloning and characterization of a second

complementary DNA for human tryptase. *J. Clin. Invest.* **86**, 864–870.[Medline]

28. Vanderslice, P., Barllinger, S.M., Tam, E.K., Goldstein, S.M., Craik, C.S. & Caughey, G.H. (1990) Human mast cell tryptase: multiple cDNAs and genes reveal a multigene serine protease family. *Proc. Natl Acad. Sci. USA* **87**, 3811–3815.[Abstract]
29. Pallaoro, M., Fejzo, M.S., Shayesteh, L., Blount, J.L. & Caughey, G.H. (1999) Characterization of genes encoding known and novel human mast ecll tryptases on chromosome 16p13.3. *J. Biol. Chem.* **274**, 3355–3362.[Abstract/Free Full Text]
30. Caughey, G.H., Raymond, W.W., Blount, J.L., Hau, L.W.-T., Pallaoro, M., Wolters, P.J. & Verghese, G.M. (2000) Characterization of human γ-tryptases, novel members of the chromosome 16p mast cell tryptase and prostasin gene families. *J. Immunol.* **164**, 6566–6575. [Abstract/Free Full Text]
31. Wong, G.W., Tang, Y., Feyfant, E., Šali, A., Li, L., Li, Y., Huang, C., Friend, D.S., Krillis, S.A. & Stevens, R.L. (1999) Identification of a new member of the tryptase family of mouse and human mast cell proteases which possesses a novel COOH-terminal hydrophobic extension. *J. Biol. Chem.* **274**, 30784–30793.[Abstract/Free Full Text]
32. Wong, G.W., Yasuda, S., Madhusudhan, M.S., Li, L., Yang, Y., Krillis, S.A., Šali, A. & Stevens, R.L. (2001) Human tryptase ϵ (PRSS22), a novel member of the chromosome 16p13.3 family of human serine proteases expressed in airway epithelial cells. *J. Biol. Chem.* **276**, 49169–49182. [Abstract/Free Full Text]
33. Min, H.-K., Kambe, N. & Schwartz, L.B. (2001) Human mouse mast cell protease 7-like tryptase genes are pseudogenes. *J. Allergy Clin. Immunol.* **107**, 315–321.[Medline]
34. Soto, D., Malmsten, C., Blount, J.L., Muilenburg, D.J. & Caughey, G.H. (2002) Genetic deficiency of human mast cell α-tryptase. *Clin. Exp. Allergy* **32**, 1000–1006.[Medline]
35. Xia, H.Z., Kepley, C.L., Sakai, K., Chellah, J., Irani, A.M. & Schwartz, L.B. (1995) Quantitation of tryptase, chymase, $F_c\epsilon RI\alpha$, and $F_c\epsilon RI\gamma$ mRNAs in human mast cells and basophils by competitive reverse transcription-polymerase chain reaction. *J. Immunol.* **154**, 5472–5480. [Abstract/Free Full Text]
36. Schwartz, L.B., Sakai, K., Bradford, T.R., Ren, S., Zweiman, B., Worobic, A.S. & Metcalfe, D. (1995) The α form of human tryptase is the predominant type present in blood at baseline in normal subject and is elevated in those with systemic mastocytosis. *J. Clin. Invest.* **96**, 2702–2710.[Medline]
37. Sakai, K., Ren, S. & Schwartz, L.B. (1996) A novel heparin-dependent processing pathway for human tryptase autocatalysis followed by activation with dipeptidyl peptidase I. *J. Clin. Invest.* **97**, 988–995.[Abstract/Free Full Text]
38. Guida, M., Riedy, M., Lee, D. & Hall, J. (2000) Characterization of two highly polymorphic human tryptase loci and comparison with a newly discovered monkey tryptase ortholog. *Pharmacogenetics* **10**, 389–396.[Medline]
39. Okayama, Y., Hunt, T.C., Kassel, O., Ashman, L.K. & Church, M.K. (1993) Assessment of anti-*c-kit* monoclonal antibody YB5.B8 in affinity magnetic enrichment of human lung mast cells. *J. Immunol. Methods* **169**, 153–161.
40. Benyon, R.C., Lowman, M. & Church, M.K. (1987) Human skin mast cells: their dispersion, purification and secretory characterization. *J. Immunol.* **138**, 861–867.[Abstract/Free Full Text]
41. McEuen, A.R., He, S., Brander, M.L. & Walls, A.F. (1996) Guinea pig lung tryptase: localisation to mast cells and characterisation of the partially purified enzyme. *Biochem. Pharmacol.* **52**, 331–340.[Medline]
42. Abe, T., Swieter, M., Imai, T., Hollander, N.D. & Befus, D.A. (1990) Mast cell heterogeneity: two-dimensional gel electrophoretic analysis of rat peritoneal and intestinal mucosal mast cells. *Eur. J. Immunol.* **20**, 1941–1947.[Medline]
43. McEuen, A.R., Sharma, B. & Walls, A.F. (1995) Regulation of the activity of human chymase during storage and release from mast cells: the contributions of inorganic cations, pH, heparin and histamine. *Biochim. Biophys. Acta* **1267**, 115–121.[Medline]

44. Ellis, K.J. & Morrison, J.F. (1982) Buffers of constant ionic strength for studying pH-dependent processes. *Methods Enzymol.* **87**, 405–426.[Medline]
45. Goldsteine, I.J., Reichart, C. & Misaki, A. (1974) Interaction of concanavalin A with model substrates. *Ann. NY Acad. Sci.* **234**, 283–296.[Medline]
46. Kornfeld, R. & Kornfeld, S. (1980) The structure of glycoproteins and their oligosaccharide units. In *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W.I., ed.), pp. 1–34. Plenum Press, New York, USA.
47. Allen, A.K., Neuberger, A. & Sharon, N. (1973) The purification and specificity of wheat germ agglutinin. *Biochem. J.* **131**, 155–162.[Medline]
48. Gallagher, J.T., Harding, M. & Dale, R.E. (1983) Sialic acid as a cell surface binding for wheat germ agglutinin. In *Lectins, Biology, Biochemistry, Clinical Biochemistry* (Bog-Hansen, T.C. & Spengler, G.A., eds), pp. 311–318. Walter de Gruyter, Berlin, Germany.
49. Debray, H., Decont, D., Strecker, G., Spik, G. & Monteuil, J. (1981) Specificity of twelve lectins towards oligosaccharides and glycopeptides related to *N*-glycosyl proteins. *Eur. J. Biochem.* **117**, 41–55.[Abstract]
50. Cummings, R.D. & Kornfeld, S. (1982) Characterization of the structural determinants required for the high affinity interactions of asparagine-linked oligosaccharides with immobilized *Phaseolus vulgaris* leukoagglutinating and erythroagglutinating lectins. *J. Biol. Chem.* **257**, 11230–11234.[Abstract/Free Full Text]
51. Segel, I.H. (1975) *Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. John Wiley and Sons, New York, USA.
52. Picard, V., Ersal-Badju, E. & Bock, S.C. (1995) Partial glycosylation of antithrombin III asparagines-135 is caused by the serine in the third position of its *N*-glycosylation consensus sequence and is responsible for production of the β-antithrombin III isoforms with enhanced heparin affinity. *Biochemistry* **34**, 8433–8440.[Medline]
53. Kirkpatrick, C.J., Jones, C.J. & Stoddart, R.W. (1988) Lectin histochemistry of the mast cell: a light microscopical study. *Histochem. J.* **20**, 139–146.[Medline]
54. Buckley, M.G., Walters, C., Wong, W.M., Cawley, M.I.D., Ren, S., Schwartz, L.B. & Walls, A.F. (1997) Mast cell activation in arthritis: detection of α- and β-tryptase, histamine and eosinophil cationic protein in synovial fluid. *Clin. Sci.* **93**, 363–370.[Medline]
55. Schwartz, L.B., Irani, A.A., Roller, K., Castells, M.C. & Schechter, N.M. (1987) Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. *J. Immunol.* **138**, 2622–2615.
56. Alter, S.C. & Schwartz, L.B. (1989) Effect of histamine and divalent cations on the activity and stability of tryptase from human mast cells. *Biochim. Biophys. Acta* **991**, 426–430.[Medline]

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